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(54) Title: DNA VACCINE AGAINST INFECTIOUS BURSAL DISEASE VIRUS

(57) Abstract

The invention concerns to the DNA–vaccines against infectious diseases of chickens first of all against infectional bursal disease (Gumboro disease). The effective material of the DNA–vaccine is a recombinant plasmid vector, which contains DNA–sequences encoding VP2–VP4–VP3 antigens, connected in well–working way with eukaryot regulatory elements providing effective gene expression in animal cells. Furthermore the invention involves new recombinant plasmid vectors and their use for immunizing chickens and for production DNA–vaccines. The invention also involves a new method for plasmid DNA purification.

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DNA Vaccine Against Infectious Bursal Disease Virus

The invention is concerning to the DNA vaccines against chicken's infectious diseases, first of all against infectious bursal disease (other name: Gumboro disease). The effective agent of the DNA-vaccine is a recombinant plasmid DNA, a vector, which contains the DNA encoding Gumboro-virus antigens and the regulating DNA-sections necessary for the gene-expression. In consequence of a vaccination by this kind of plasmid DNA-vaccine a Gumboro-specific antigen-protein will be expressed in the vaccinated animal and as a result of this a humoral immune answer is arising in the chickens, which provides protection against infectious diseases. The DNA-vaccine involved in this invention can be used for vaccination chickens against infectious diseases, first of all against infectious bursal disease.

Furthermore the new recombinant plasmid vectors and their application for immunizing chickens against infectious diseases, as well as their usage for producing the above DNA-vaccines are all involved as the subject of the invention.

The Gumboro-virus is the virus of the infectious bursal disease (infectious bursal disease virus, hereinafter: IBDV), which is belonging to the Birnaviridae family, and damages the Bursa Fabricii (inflammation, phthisis). The virus is mainly spreading in meat-chicken farms and attacks the young broiler chickens, but it is infectious to hen stocks too. All the known pathogen IBDV strains are belonging to the same serotype. The replication of the IBDV is happening in the limphoid organs, mainly in the Bursa Fabricii. Immunodeficiency is developing in the chickens infected by IBDV, which is cause by the lack of B-lymphocyte precursors. This increases the sensitivity against other pathogens such as e.g. Salmonella enteriditis, and decreases the number of the surviving animals (Lutticken, 1997). In addition the IBDV infection decreases the effectivity of the vaccination against fowl pox, Marek-disease and infectious bronchitis. Recently it was found, that the IBDV infection induces apoptosis both in vivo and in vitro (Tam and Moon, 1996; Vasconselos and Lam, 1995), which can be assigned to the immunosupressive effect of the IBDV. This disease occurs in the big chicken farms throughout the world and causes serious losses of the stocks. In the recent years the number of the high-mortality IBDV-epidemics were

uniformly increasing (Brown and Skinner, 1996).

The genom of the IBDV virus consists of two double-stranded RNA-segments, their size is about 3400 and 2900 base pairs (Dobos et al., 1979). The smaller segment (segment B) encodes the VP1 protein and its own RNA-polimerase, while the bigger segment (segment A) ecodes a 115 kD precursor polypeptide in a single big opened reading frame, which components are processing into matured VP2, VP3 and VP4 structure-proteins (Hudson et al., 1986). The segment A also encodes an own 15 kD VP5 protein, of which function is not known yet. The VP2 and the VP3 are the big structure-proteins of the virion. The VP2 constitutes the dominant protective effect immunogen. It has considerable genetic heterogenity among the IBDV strains (Jackwood and Sommer, 1998) and contains epitopes reliable for inducing neutralizing antibodies (Becht et al., 1998; Heine et al., 1991). VP3 can be regarded as a group-specific antigen, while VP4 is a protease, which participates in the formation of the precursor polypeptide (Jagadish et al., 1988). The total nucleotide-sequence of the segment A was already determined at numerous strains (Vakharia et al., 1994; Brown and Skinner, 1996).

Nowadays such kind of vaccines are applied against the IBDV infection, which contain inactivated or weakened living virus. E.g. in the Hungarian patent No. 203781 the production of an inactivated vaccine against infectious Bursal disease is described, in the Hungarian patent No. 203780 the production of a liofilized vaccine is described which contains living virus. In the announced patent No T/67324 there is described a vaccine against the Gumboro disease, which contains effective, immunizing quantity of living, weakened, mid-level virulency IBD virus. It was observed in the USA, that against certain variant virus strains, such as Delaware or GLS, the expensive vaccinations are not effective. In addition recently very virulent variant IBDV strains has appeared, which also made necessary to develop new vaccines and vaccinating strategies. The vaccines effective against these strong virulency virus strains are made of that kind of strain, which has considerable residual virulency, so thats why they can cause immune-supression. Recombinant IBDV vaccines providing protective immunity for chickens were worked out, which were based on the expression of the antigen, e.g. in yeast (Macreadi et al., 1990), in baculovirus (Pitckovski et al., 1996), in adenovirus (Sheppard et al., 1998) and in fowlpox

virus (Bayliss et al., 1991; Heine and Boyle, 1993). Nevertheless the production of these vaccines is very expensive.

Because of this there is necessary to develop such a new kind of vaccine, wich - besides its higher efficiency - could simplify the IBDV vaccine production and reduce the production costs. Recently several publications described the development of effective immuno-answer in animals immunized by purified DNA. The development of the immunization by DNA inoculation could ba a unique method for the defence of animals against infectious diseases, as e.g. rabies, papilloma, malaria and hepatitis B (see the last in Robinson et al., 1995). The defence of chickens against influensa virus (Robinson et al., 1993) and against Newcastle-disease virus (Sakaguchi et al., 1996) using plasmid DNA for vaccination opened new perspecive in the development of new generation vaccines.

The purpose of the invention is to work out DNA-vaccine against chicken's Gumboro disease, which could be produced with low costs and besides providing high effectiveness and harmlessness.

We've solved this task with a new recombinant plasmid DNA. This plasmid DNA constitutes the basis of the DNA-vaccine, which is a recombinant expression plasmid vector, in which the DNA sequences encoding the IBDV antigens were inserted under such kind of eukaryot regulatory elements, which are able to provide the effective expression as well as overexpression of these genes in animal cells.

Accordig to the above mentioned things the expression plasmid vector constitutes the subject of the invention, which contains in animal cells DNA sequences encoding IBDV VP2-VP4-VP3 antigens which are linked by operative way with eukaryot regulating elements providing effective gene expression.

In the advantageous realization method the recombinant plasmid vector has bacterial origin, preferably of E. coli origin. The vector is human citomegalovirus, adenovirus and contains SV40 transcription regulator elements, which provides the effective expression of the genes of foreign origin in animal cells. The promoter and the enhancer has cytomegalovirus origin, the leading sequence is the tripartite leader sequence of the adenovirus and the polyadenilation is provided by the SV40 polyadenilation signal-sequency. The section providing the adenovirus splicing - which also a very important

regulating element – is inserted besides the promoter. At other realization way of the invention another eukaryot regulating elements may be used, which ones are known by an expert skilled in this field. In all experiments the circular form of the plasmid DNA was used, as – in contrast with the chicken vaccinating data published so far (linear plasmid DNA-vaccine against Newcastle disease, Sakaguchi et al., 1996) – we've found essentially lower the immunizing effectivity of the linear form of the IBDV DNA.

Furthermore the invention is concerning the use for immunizing chickens against infectious diseases of the recombinant plasmid vectors involved in the patent, especially against the infectious Bursitis disease (Gumboro disease).

That kind of the use is considered as the subject of the invention, in which the above mentioned recombinant plasmid vectors are used for producing DNA-vaccines.

Accordingly the invention furthermore covers DNA vaccines, which contain the recombinant plasmid vector and physiologically acceptable carrier media involved in the invention. These DNA-vaccines are applicable for the protection of chickens against infectious diseases, first of all against the infectious bursal disease. The vaccine involved in the invention has the special advance opposed to all the other existing effective IBDV vaccines, that besides its high effectivity it is not immunosupressive. So the DNA-vaccine involved in the invention can provide good defence against the other infectious diseases by defending the chickens against the infectious bursal disease.

The vaccine products involved in the invention can be introduced parenterally or orally. Favourably they can be introduced parenterally, e.g. subcutan, intradermally, intramuscularly, intraperitoneally, intraocularly e.t.c., the best favurables are the intramuscular and intraperitoneal methods.

The products involved in the invention used for parenteral introduction purpose are occuring in a pharmcologically acceptable carrier – favourably in an aquatic carrier – as a solutions or a suspensions of the plasmid. Water, buffer-solution, salt-solution or other known carriers can be used as aquatic carriers. Advantegously PBS-solution or physiological sodium-chloride solution can be used. In given case, the specific can contain a usual adjuvant also. In addition the product can contain pharmacologically acceptable additives, e.g. buffer materials and inorganic salts, in order to ensure the normal osmotic

pressure and/or the effective liofillization. Sodium-, potassium-, salts, e.g. chlorides and phosphates, saccharose, glucose, protein-hydrolizates, dextrane, polyvinile-pirrolidon or polyethylene-glycol can serve as that kind of additives. The products could be sterilized by usual techniques, e.g. by sterile-filtering. In this form, the product can be liofillized or filled directly and only necessary to mix it up with a sterile solution before using.

An expert can receive the adequate auxiliaries anf formulas from standard handbooks, such as "Remington's Pharmaceutical Sciences", 1990.

It is possible to introduce the pharmaceutical products involved in the invention using liposomes, which are stabilizing the plasmids. So, the use of the cation liposoma regulated gene-transfer technique (Gao and Huang, 1995) could effectively decrease the DNA-vaccine quantity necessary for inducing the protection.

In the vaccine-product involved in the invention, the effective amount of the plasmid vector could be changed in wide ranges. The amount of the plasmid is mainly depending on the method of the introducement and the given form of the packaging. The introduceable quantity of the plasmid is generally between 1,0 μg and 1000 μg, adventageously between 15 μg and 800 μg.

Below we describe the figures:

Figure 1.: Map of the pLVP2 recombinant plasmid

Figure 2.: Map of the pWGP2 recombinant plasmid

Figure 3.: Map of the pWVP9 recombinant plasmid

Figure 4.: Map of the pLVP2-4 recombinant plasmid

Figure 5.: Map of the pLVP5 recombinant plasmid

We use the below shortenings on the plasmid-maps:

lacZ = E. coli β-galactosidase

lacI = repressor

Ap = ampicillin resistancy

ORI = bacterial origo

VP2, VP3, VP4 = genes encoding gumboro-virus (IBDV) antigens

CMVpro = cytomegalovirus promoter

Avtl = adenovirus splicing DNA-section

polyA = polyadenilating DNA-section

The cutting spots of the restriction enzymes are signed on the outer part of the circle; B = BstXI restriction enzyme.

In the below examples the following materials and methods were used, except if there are others mentioned:

Cloning IBDV cDNA and identification of the sequency of genes ecoding antigens

The methods for molecular cloning were essentially used as described by Sambrook et al., 1989. The purification of the IBDV D78 strain (Intervet International B.V., the Netherlands), the isolation of the viral DNA, the reverse transcription, the cloning of the A segment and the identification of the nucleotide-sequences encoding the *vp2-vp4-vp3* polyprotein were made according the methods described by Vakharia et al. (Vakharia et al., 1992 and 1994).

The methods for purifying the native IBDV GP40 strain (which was provided by the Phylaxia Sanofi, Budapest), the isolation and cloning of the viral RNA and the identification of the nucleotide-sequence of the whole *vp2* gene were made manly according to the methods published by Vakharia et al. in 1992. In details, we denaturated the GP40 double-stranded RNA and carried out reverse transcriptase-polymerase chain reaction (RT-PCR), in which we used rTth polymerase (Boehringer Mannheim). For the RT-PCR reaction we synthetized the primers listed in Table 1. We marked the sequence of the primers according to the structure of the IBDV D78 strain's genom.

vp2 in the sequence-list as the sequence No 1. The amino-acid sequence of the VP2 protein is given in the sequence list as sequence No 2., where the amino-acid remnanats are marked by the internationally accepted single letter codes. We found two differences in the structure of the GP40 VP2, when compared to the VP2 protein of the D78 strain: aa42 (A/T) and the aa125 (D/N).

B) Production of plasmids

The starting pWS4 plasmid (Sheay et al., 1993) was given by Dr. R. Dornburg. This plasmid vector contained CMV and adenovirus regulator elements, which provides very effective gene-expression in animal cells. We produced two constructions: such kind of plasmids, which contain the DNA encoding the VP2 antigen of the virus (pLVP2, pWGP2, pWVP9), and that kind of plasmids, which contain DNA encoding all three of antigens, VP2, VP4 and VP3 (pLVP2-4, pLVP5). The genes were produced from D78 and GP40 IBDV strains. We constructed that kind of plasmids, which contain gene isolated from the domestic GP40 strain besides the genes origin from the D78 strain. An important characteristic of the pLVP5 and pWVP9 (this is marked as pLWP9 too), that in this case the sequences which are unnecessary for the gene expression were successfully removed.

For producing the pLVP2-4 expression plasmid, the blunt-ended StuI-NsiI DNA-fragment (3,22 kb) of the D78 strain was inserted into the blunt-ended NotI-PstI sites of the pWS4 plasmid. The pLVP5 construction origins form the pLVP2-4 plasmid in such a way, that we deleted that redundant DNA-segment, which contained the multiple restriction places on the 3' end of the IBDV sequences. We produced the the pWGP2 plasmid so, that we built the XbaI-HindIII fragment of the GP40 *vp2* gene into the XbaI-HindIII sites of the pWS4. We produced the pWVP9 plasmid was produced by the deletion of the redundant flanking polylinker sequences. The pLVP2 plasmid was constructed in such a way, that the 1,4 kb StuI-SalI fragment of the D78 *vp2* gene was inserted into the BstXI and SalI sites of the pWS4. We verified the structures of all the plasmids by sequncing them (Vakharia et al., 1994). The plasmid maps are shown on Figures 1-5.

Table 1

Primer	Sequence	Size	Genom site
8 F	5' -attacccgggaaatgacaaacctgcaagatcaaaccc	37 bp	95-120
9R	5' -atatgaattctaccttatggccctgattatgtctttg	37 bp	1451-1427

We were cloning the PCR-fragments carrying the *vp2* gene into the SmaI-EcoRI sites of the pBluescript II SK vector (Stratagene). The recobinant plasmid containing the GP41 *vp2* gene - which was produced by this method - was named as pBVP17, it was alalysed by restriction enzymes and was characterized by its whole nulcleotide-sequence, which was determined by PCR cycle sequencing. We used the primers listed in Table 2 for sequencing the GP40 *vp2* gene.

Table 2

Primer	Sequence	Strand
M13REV	CAG GAA ACA GCT ATG ACC	reverse
M13FOR	TGT AAA ACG ACG GCC AGT	forward
vp2 (252-271)	ATG CTC CTG ACT GCC CAG AA	sense
vp2 (636-655)	GAT TAC CAA TTC TCA TCA CA	sense
vp2 (941-963)	C TCC AAA AGT GGT GGT CAG GCA G	sense
vp2 (1184-1164)	CAT GGC TCC TGG GTC AAA TCG	antisense

We fractionated the pBVP17 clone origin PCR cycle sequencing products by electophoresis on carbamide containing 6 % polyacrilamide-gel, with an automatic DNA-sequencer. The sequence data were investigated thoroughly, that whether they are ambiguous, and - where it seemed to be necessary - the sequencing reaction, the fractionating and the analysis of the reaction-products were repeated. The data are showing, that the encoding sequence of the of the *vp2* gene consists of 1356 bases and encoding a protein, the VP2, which contains 452 amino acids. We give the nucleotide-sequence of the

C) Purification of plasmid DNA

It is necessary to produce very clean DNA for the vaccination experiments, as the pollutants, e.g. polysaccharides could cause serious side-effects in the immunized animals. Therefore we had worked out a new, effective plasmid-purification method, which allows to produce 1-10 mg of pure DNA suitable for vaccination, from 0,5-2,0 litres of E. coli culture.

In more detail, E. coli XL1-Blue cells (Stratagene) were transformed with expression DNA-plasmids involved in the invention, the transformed cells were cultured on LB culture-medium, to which ampicillin was added. The cells were collected and exposured in 0,1-0,2 n NaOH and 0,5-1,0 % SDS solutions. We treated the lizatum with K-acetate (pH 8,4) and removed the precipitated material. We precipitate the nucleic acids (DNA and RNA) from the solution with 0,6 volume equivalent izopropanol. After centrifugation the precipitation was dissolved in TE-buffer and the RNA was removed by incubating with RN-ase. After centrifugation the DNA solution was treated by 0,1 M $MgCl_2$. Hereafter the DNA was treated with phenol, which was later removed using chloroform/izoamilalcohol mixture. We precipitated the DNA with ethyl-alcohol, then dissolved in TE-buffer and finished the DNA purification with a single CsCl-gradient centrifugation. The OD of the concentrated, purified DNA solution was measured, generally we resulted solutions of 0,6-6 mg/litre concentration.

The DNA produced this way was used for vaccination in sterile 0,9 % NaCl solution or in PBS buffer solution.

The advance of the method is that as the DNA was purified essentially more thoroughly, it made possible to earn the necessary quantity DNA with a single CsCl-gradient centrifugation. In other known methods there is a necessity to repeat this step (accordingly to make CsCl-gradient cenrifugalization twice), which has much more time-requirement, more expensive and in addition decreases the DNA yield. Another known settling is the purification on a QIAGEN-column, which is a more simple but much more (2-3 times) expensive.

D) Vaccination experiments

One-day or 3-4 weeks old SPF (specified pathogen free) chickens were used for the vaccination experiments. During the whole experiment the chickens were kept in separate isolation units in order to prevent the chickens used for vaccination from any IBDV infection and that these chickens be sero-negative ones to the IBDV. For evaluating the effectiveness of the gene-immunization with *vp2* or *vp2-vp4-vp3* genes, the one-day-old SPF chickens placed into the isolator were inoculated twice with the interval of 14-17 days by intramuscular and intraperitoneal way, with 50+50 μg equivalemt plasmid DNA in phosphate-buffered salt solution (PBS), namely altogether with 100 μg/chicken quantity. We purified the plasmid DNAs from E. coli according to the above described method. The control group got only the starting plasmid in PBS. The chickens were vaccinated with the plasmid-DNAs expressing the VP2 antigen protein as well as the VP2-VP4-VP3 polypeptide. On the day 14-16th following the second vaccination we infected the chickens with the CVL 52/70 virulent IBDV strain (10^3 x EID_{50} = egg infective dose 50) and simultaneously we collected blood samples from the animals. The blood-sera originated from the blood samples were kept on 56 °C for 30 minutes for inactivate the specific antibodies, and we determined the titer of the virus neutalizing antibodies in a virus-neutralizing test. The resulted numerical data are provided in Example 4., see below the summary of the results.

Results

Surprisingly we found, that the vaccination with the plasmid DNAs encoding the VP2 antigen, so the vaccination with the pWGP2 or pLVP2 plasmids encoding GP40 or D78 VP2 resulted non-measurable antibody level, which could be assigned to that fact, that the correct conformation could be formed only in the presence of the other proteins. Namely the host protective epitopes in the VP2 are strongly conformation dependents, and the correct folding structure is necessary for achieving a protective immuno-answer (Azad et al., 1991). So, in a transient expression system, the relatively low level of the plasmid

induced antigen and the lack of the strongly conformation dependent epitopes provides a possible explanation of the uneffective vaccination with VP2 plasmids. Accordingly the vaccination with these plasmids could not provide satisfactory protection for the chickens against virulent IBDV strains.

On the other hand we'd found, that the vaccination made with the pLVP2-4 and pLVP5 plasmids - which are expressing the VP2-VP4-VP3 precursor polyprotein - is much more effective in chickens against IBD, which could be assigned to the better positioning of the epitopes on the correctly processed VP2 and VP3 proteins. The correct processing of the recombinant precursor protein was already detected in chicken cells (Heine and Boyle, 1993), and we supposed, that during the DNA inoculation the synthetized 115 kD VP2-VP4-VP3 precursor protein is self-processed into three autentic proteins. The VP2 and VP3 are the main structure proteins of the virion and these can form virus-like particules, of which immunogenity is much higher than the soluble single proteins. In addititon the VP3 could contribute to the immuno-answer of the chickens, as it can be considered as a group-specific antigen. Other researchers did not succed in detect seroconversion and immunity in chickens with such a kind of fowlpox virus vector, which encdes the VP2-VP4-VP3 precursor polyprotein, although the recombinant precursor polyproteinhas been self processed (Heine and Boyle, 1993).

Furthermore we found that the deletion of the unnecessary sequences for the gene-expression from the regulator regions, heavily improved the expression of the antigen. Consequently the removal of the polylinkers from the pLVP2-4 construction, the resulted pLVP5 provided better immunization for the chickens (see Table 4).

Direct relation was found between the IBDV-specific (VP2) antibody titer and the immunitiy in immunized chickens, and in the VN-test those birds became immunized, which had at least 1:40 - 1:60 antibody titer (Nakamura et al., 1994). During our experiments we found, that all the protected bird had improved IBDV specific antibody titer. Nevertheless in several cases had developed a kind of antibody answer, which did not provided immunity. Surprisingly three chickens - against their high, 1:600 - 1:3200 antibody titer (see Table 5) - did not become protected against the disease. Deciding this, it

makes necessary the further examination of the cellular immuno-answer's role in the protective immunization against IBD.

Below we describe detailed the invention without limiting the protection range.

Example 1

Constructing the plasmids containing the *vp2* gene (pWGP2, pWVP9 and pLVP2)

pWGP2:

We treated 1 μg pWS4 starting plasmid with 10 units of XbaI and HindII restriction enzymes and isolated the DNA from the gel after a 0,8 % agarose gel-electrophoresis.

We treated 1 μg plasmid encoding the VP2 protein of the GP40 strain with 10-10 units of XbaI and HindII restriction enzymes and the DNA fragment of about 1,4 kb size was isolated from the gel with gel-electrophoresis. The two DNA was mixed and treated with DNA-ligase. The resulted plasmid DNA was marked as pWGP2, its map is shown on Figure 2.

pWVP9:

We treated 1 μg pWGP2 DNA with 10-10 units of BstXI and Pvu restriction enzymes and isolated the DNA from the gel after a 0,8 % agarose gel-electrophoresis. The DNA was treated with DNA-ligase.

In the second step the above produced DNA (1 μg) was treated with 10-10 units of HindIII and KpnI restriction enzymes and respectively with Klenow enzyme. The DNA was isolated from the gel after a 0,8 % agarose gel-electrophoresis and was treated with 100 units of DNA ligase. The name of the produced DNA is: pWVP9, its map is shown on Figure 3.

pLVP2:

1 μg pWS4 (starting vector) was treated with 10 units of BstXI and SalI restriction enzymes, the DNA was isolated from the gel after a 0,8 % agarose gel-electrophoresis.

We treated 1 μg plasmid encoding the VP2 protein of the D78 strain with 10-10 units of StuI and SalI restriction enzymes and the DNA fragment of about 1,4 kb size was isolated from the gel with gel-electrophoresis. The two DNA was mixed and treated with 100 units of DNA-ligase. The name of the produced plasmid is: pLVP2, its map is shown on Figure 1.

Example 2

Constructing the plasmids containing the vp2-vp4-vp3 genes (pLVP2-4, pLVP5)

pLVP2-4:

1 μg pWS4 (starting vector) was treated with 10 units of NotI and PstI restriction enzymes, the DNA was isolated from the gel after a 0,8 % agarose gel-electrophoresis.

We treated 1 μg plasmid encoding the VP2-VP3-VP4 proteins of the D78 strain with 10-10 units of StuI and NsiI restriction enzymes and the DNA fragment of about 3,22 kb size was isolated from the gel with gel-electrophoresis. The two DNA was mixed and treated with 100 units of DNA-ligase. In case of necessity the fragments were treated with Klenow polymerase in order to make the ends blunt. The name of the produced plasmid is: pLVP2-4, its map is shown on Figure 4.

pLVP5:

We treated 1 μg pLVP2-4 plasmid with 10-10 units of EcorV and KpnI restriction enzymes, than made the KpnI end blunt using Klemow enzyme. The DNA was purified

with 0,8 % gel-electrophoresis and was treated with DNA-ligase. The name of the produced plasmid is: pLVP5

Example 3

Purification of plasmid DNA

We took a single isolated colony from the freshly streaked plate which contained an E. coli XL1-blue strain (Stratagene) transformed with expression plasmid factors mentioned in the Examples 1 and 2, inoculated it into an LB culture-medium which containing 5 ml ampicillin, then it was incubated during one night on 37 °C. On the next day 2.5 ml of this culture was used for inoculating 500 ml LB culture-medium containing 50 µg/ml ampicillin, and we incubated this for 12-16 hours on 37 °C using vigorous shaking. We collected the cells by cetrifugation (6000x g for 20 minutes, 4 °C). The cell pellet was resuspended in 5.0 ml 50 mM Tris-HCl buffer, pH 8.0, 10 mM EDTA at room-temperature. Then equal volume (5 ml) of 0.2 n NaOH, 1 % SDS was added to cell-suspension, carefully mixed and left for 30 minutes of incubation at room temperature. Then the cells were lysated with 5 ml of 1.32 M Ka-acetate buffer, pH 4.8 and the mixture was vigorously shaken and centrifuged at 12,000x g for 20 minutes at room temperature. The supernatant was cleared by filtering it with paper filter, and then 0.6 volume of isopropanol was added for 5 minutes at room temperature to precipitate the nucleic acids (DNA and RNA). After a 15 minutes centrifugation at 12,000x g the pellet was dissolved in 2 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The own RNA was removed by ribonuclease A treatment (Sigma, 10 mg/ml) with the final concentration of RNase 75 µg/ml incubated for 45 minutes at 37 ?C. Following this the solution of $MgCl_2$ was added to the final concentration 0.1 M and the solution was incubated for 15 minutes at 0 °C, then it was centrifuged for 10 minutes at 12,000x g at +4 °C. The supernatant (DNA solution) was phenol-excracted with the equal volume of phenol and the water phase was removed by a 10 minutes centrifugation (12,000x g) at room room temperature. The phenol phase was treated with the 0.5 volume of TE buffer in a similar way and both TE phases with the DNA were

combined, extracted with chlorophorm/isoamyl alcohol (24:1) and centrifuged. The upper water phase was removed and thoroughly mixed with equal volume of chlorophorm and then centrifuged as described above. This procedure was performed twice. The upper phase of the DNA solution was precipitated by adding 3M Na-acetate buffer, to the final concentration of 0.3 M, and then two volumes of ice-cold EtOH were added. The mixture was thoroughly mixed and the centrifuge tube was kept at -20 °C for 20minutes. The DNA was collected by centrifugation (12,000x g, 10 minutes, 0 °C), then the DNA pellet was washed with two cycles of centrifugation in cold 70 % EtOH, and the DNA was dissolved in TE buffer. The DNA was further purified on CsCl-EtBr gradients; the EtBr was removed by multiple n-butanol extraction. After 2.5x dilution with sterile H_2O, the resulting DNA was EtOH-precipitated and the DNA was resuspended in sterile 0,9 % NaCl for injection. The concentration and purity of each DNA preparation was determined by OD 260/280 ratio which was in the range 1.9-2.0. Using this effective plasmid purification method it became to purify 1-10 mg of DNA from 0.5-2 litres of E. coli culture, which DNA preparation is directly suitable for vaccination.

Example 4

Vaccination experiments

(A) The immunizing effect of the plasmids produced according to Example 1 – which ones are encoding only the VP2 antigen of the virus – was compared to the immunizing effect of the pLVP2 plasmid according to Example 2, which encodes all the three proteins of the virus (VP2, VP4, VP3). The vaccination of the chickens were performed according to the above described method (see: vaccination experiments). The chickens kept in the isolator were divided into four groups, and all groups were vaccinated with one of the four plasmids, namely with pWGP2, pLVP2, pLVP2-4 or pWS4. The first two plasmid expresses the VP2 protein of the GP40 respectively the D78 strains, tha pLVP2-4 expresses the precursor VP2-VP4-VP3 polypeptide, and the pWS4 vector served as control. On the 14th day following the second injection the chickens were infected with virulent CVL52/70

IBDV strain (10^3xEID$_{50}$), and blood samples were collected from the animals. The sera taken from the blood samples were analyzed for the presence of IBDV specific antibodies with 1:4 - 1:512 dilution in virus-neutralizing (VN) test. For this we used primer SPF chicken-embryo fibroblast cells and the IBDV strain marked 133-593 TCID$_{50}$ (tissue culture infective dose 50) GP. The surviving chickens were euthnized 6-9 days after the infection and their bursae were removed. We verified the protection against the infectious bursal disease with the macroscopic and/or hystopathological examination of the bursae. The results of these experiments are shown in Table 3.

Table 3

Immunization chickens with plasmids expressing VP2 antigen
or with VP2-VP4-VP3 polypeptide and their infection with IBDV

Plasmid	Number of chickens	Macroscopic bursal lesions (positive/negative)	VN test (133xTCID$_{50}$)
pSW4	11	11/0	11x < 1:4
pWGP2	12	12/0	12x < 1:4
pLVP2	7	5/0	5x < 1:4
		1/0	1:22,6
		1/0	1:76
pLVP2-4	7	3/0	3x < 1:4
		1/0	1:6
		1/0	1:64
		1/0	1:361
		0/1	1:192
Infected control	10	10/0	10x < 1:4

The data of Table 3 show, that the vaccination made with plasmids pWGP2 and control pWS4 did not resulted immuno-answer. We found improved VN test immuno-

answer only in two pLVP2 treated chickens, but the bursae of all chickens showed the signs of disease. But the vaccination with the pLVP2-4 plasmid produced positive results: 4 animals proven seropositive among the 7 ones, and one of them was protected pathologically and hystopathologically too. Surprisingly the bird showing the strongest immunoanswer did not get protection against the bursal lesions. We concluded from these experiments that the vaccination with the plasmid construction expressing the VP2-VP4-VP3 polyprotein is far better then the vaccination with the plasmid expressing only the VP2 antigen. It is necessary to consider, that a day-old chickens are hardly immunizable. In addition the strength of the infection was 10^3xEID_{50}, ten times higher, in opposite to the normally used 10^2xEID_{50} level.

(B) For the better evaluation of the effectiveness of the vaccine involved in the invention, 4 week old SPF chickens were vaccinated twice with 14 days interval with the pLVP5 DNA-plasmid (see Example 2), which also expresses the VP2-VP4-VP3 polyprotein, but does not contain the DNA sections unnecessary for the expression, as described above. Fourteen days following the second injection blood samples were collected, and the chickens were infected by stripping their eyes with 362 x EID_{50} virulent CVL52/70 IBDV strain. The birds got 15.6 μg or 150 μg DNA accordig the above described two intervention methods, hence altogether 31.2 or 300 μg plasmid DNA. The result of these experiments are summarized in Table 4.

Table 4

Vaccination of chickens with 31.4 or 300 µg pLVP5 plasmid and their infection with IBDV

No of vaccinated chickens	Plasmid/chick (µg)	Bursa lesions		VN-test ($593xTCID_{50}$)
		Macroscopic pos./neg.	Hystopathology	
17	pLVP5: 2x15.6	17/0	ND	17 x < 1:10
1	2x15.6	1/0	ND	1:10
1	2x15.6	1/0	ND	1:15
1	2x15.6	1/0	ND	1:40
1	2x15.6	1/0	ND	1:60
1	2x15.6	1/0	ND	1:80
1	2x15.6	0/1	neg	1:67
1	2x15.6	0/1	neg	1:160
1	2x15.6	-/+	neg	1:2,149
5	pLVP5: 2x150	5/0	ND	5 x < 1:20
1	2x150	1/0	ND	1:20
2	2x150	2/0	2xpos	2 x < 1:30
1	2x150	1/0	ND	1:120
1	2x150	1/0	neg	1:190
1	2x150	1/0	neg	1:240
1	2x150	0/1	neg	1:960
1	2x150	-/+	-/+	1:960
1	2x150	-/+	pos	1:1,280
14	pWVP9 : 2x150	14/0	14xpos	14 x < 1:10
15	Non-vaccinated control	15/0	ND	15 x < 1:2

neg = 100 % protection; ND = not examined

The data of Table 4 are showing, that from the chicken group immunized with 2x150 µg DNA, 9 from the 14 animals (64 %) developed different level of virus neutralizing antibody titer 14 days after the second injection, and in addition, 3 animals form this group (21 %) were entirely, and one of them was partially protected against the infection. In one chicken, besides the high antibody titer, we found bursal lesions. The treatment with 2x15.6 µg DNA proved less effective, as only 8 (32 %) of the 25 vaccinated

animal gave antibody answer and 3 of them (12 %) was protected. In spite of this none of the control chickens produced IBDV specific antibodies and protection against the infection.

(C) For the purpose to prove the effectiveness of the immunization, we increased the total amount of the DNA-vaccine up to 800 μg/animal. Three week old SPF chickens were immunized with a single intramuscular (200 μg) and intraperitoneal (200 μg) injection, altogether with 400 μg DNA-plasmid per chicken, and we repeated the injection with the same dose in 14 days (altogether 800 μg). The infection was performed on the 16th day after the second immunization with 10^3xEID_{50} IBDV CVL52/70 strain. The resulted data are shown in Table 5.

Table 5

Vaccination of chickens with 800 μg pLVP5 and infection with IBDV

No of chickens (n)	VN-test on day 14th (333x$TCID_{50}$)	VN-test on day 30th (333x$TCID_{50}$)	Bursa lesions (hystopathology)
1	≤1:2	1:800	neg
1	≤1:2	≤1:2	pos (+/-)
1	≤1:2	1:3200	pos
1	≤1:2	1:300	neg
1	1:6	1:600	pos
1	≤1:2	1:600	neg
1	≤1:2	≤1:2	pos (+/-)
1	1:24	1:200	neg
1	≤1:2	≤1:2	pos
1	≤1:2	≤1:2	pos (+/-)
1	≤1:2	≤1:2	pos
pWS4 n = 5	≤1:2	≤1:2	pos
PBS n = 5	≤1:2	≤1:2	pos

neg = 100 % protection, +/- = partial protection

The data of Table 5 are shiwing, that the vaccination of the chickens with pLVP5 in this case induced seroconversion in six birds (55 %), and provided entire protection in 4 animals (36 %). It provided effective protectiof for most of the birds against the virulent IBDV virus, whilst in the control experiments performed with pWS4 and PBS the chickens did not show any immuno-answer and protection.

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CLAIM POINTS OF THE INVENTION

1. Recombinant expression vector plasmid, which contains DNA-sequences encoding VP2-VP4-VP3 antigens, connected in well-working way with eukaryot regulatory elements providing effective gene expression in animal cells.

2. Recombinant plasmid vector according to the claim point 1., which contains human cytomegalovirus direct early enhancer and promoter as regulatory elements, threepartide adenovirus leader-sequency, SV40 polyadenilation signal-sequency, and an adenovirus splicing sector between the promoter nad the SV40 signal-sequency.

3. Recombinant plasmid vector according to the claim points 1. and 2., where the plasmid vector has bacterial – advantageously E. coli – origin.

4. The pLVP2-4 plasmid according to Figure 4.

5. The pLVP5 plasmid according to Figure 5.

6. The use of any plasmid vectors according to the claim points 1-5. for immunization of chickens against infectious diseases.

7. The use for immunization of chickens against infectious bursal disease according to the claim point 6.

8. The use of plasmid vectors according to any of the claim points 1-5. for producing DNA-vaccine against infectious diseases of chickens.

9. The use according to the claim point 8. for producing DNA-vaccine against infectious bursal disease.

10. Vaccine for protecting chickens against infectious diseases, which contains a recombinant plasmid vector according to any of the claim points 1-5. and physiologically accepteble carrier medium/media.

11. Vaccine according to the claim point 10. for protecting chickens against infectious bursal disease.

12. Vaccine according to the claim point 11. which is suitable for parenteral intervention.

13. Method for DNA purification, characterized as

(i) the E. coli XL1-Blue cells transformed by the plasmid DNA to be purified are cultured on LB culture-medium supplemented with ampicillin,

(ii) the cells are lysated, the lysate is treated by K-acetate and the precipitated material is removed,

(iii) the DNA and the RNA are precipitated from the solution by isopropanol, after centrifugation the precipitation is dissolved in TE buffer and the RNA is demolished by RN-ase and centrifuged,

(iv) the DNA solution is treated with MgCl solution and then with phenol, which is removed later using chlorophorm/ isoamyl alcohol mixture,

(v) the DNA is precipitated with ethil-alcohol, dissolved in TE-buffer and purified by CsCl-gradient centrifugation.

14. The method described in claim point 13., characterized with, that any plasmid DNA is purified accordint to the claim points 1-5.

Fig. 1

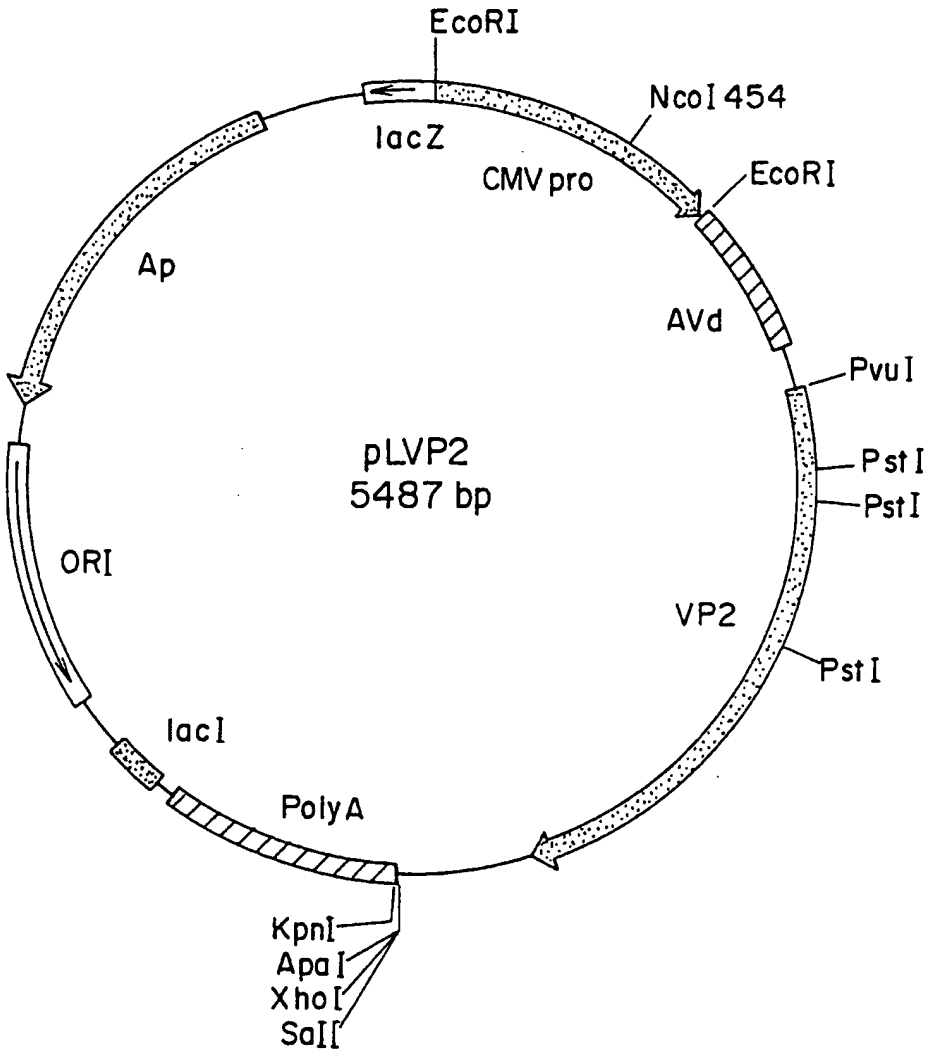
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Fig. 2

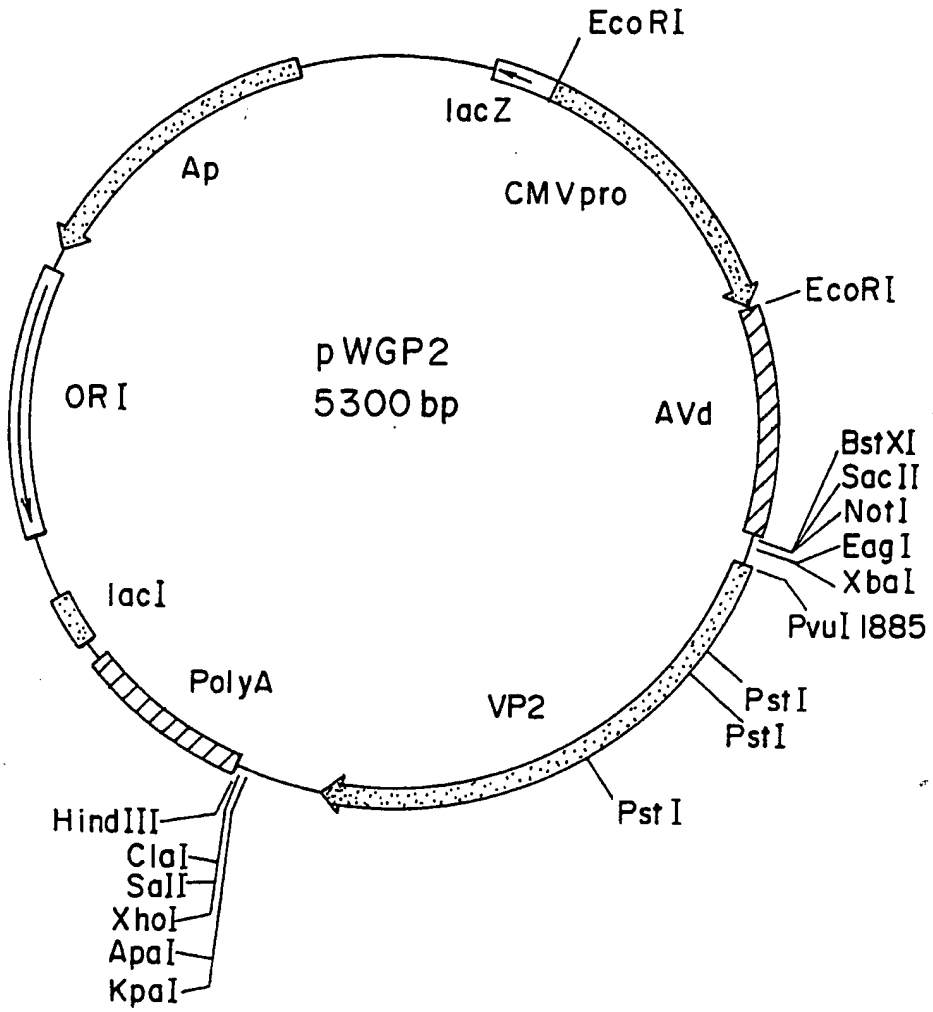
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Fig. 3

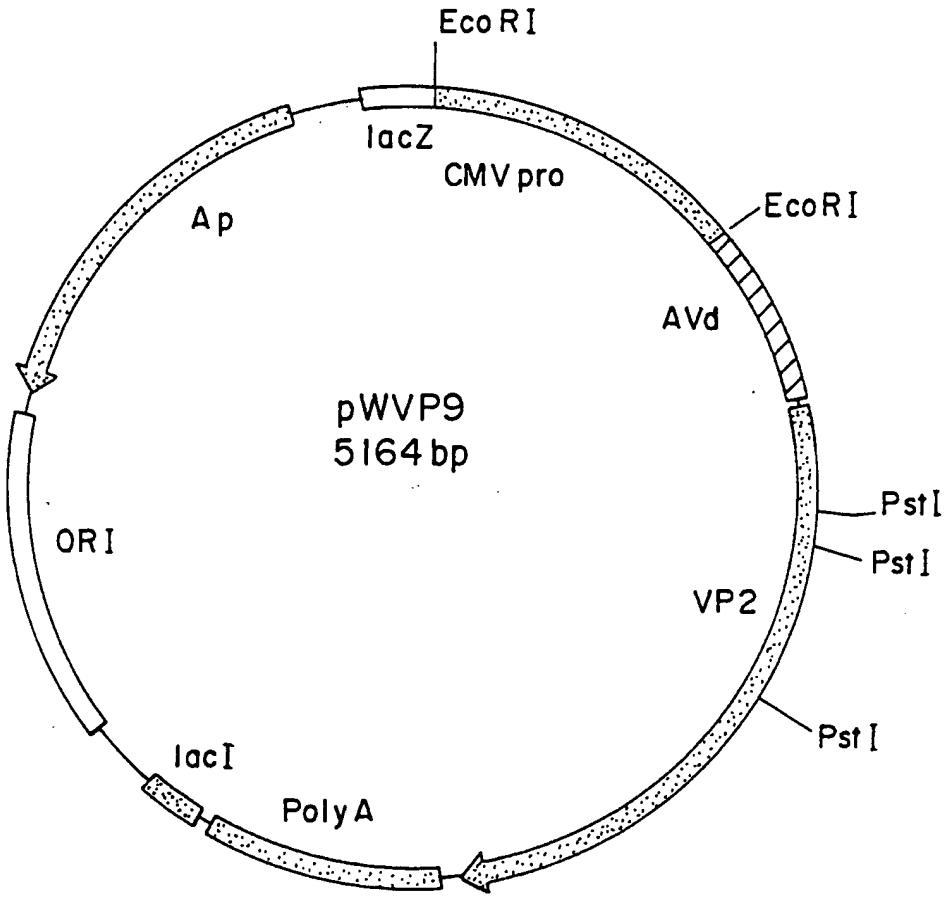
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Fig. 4

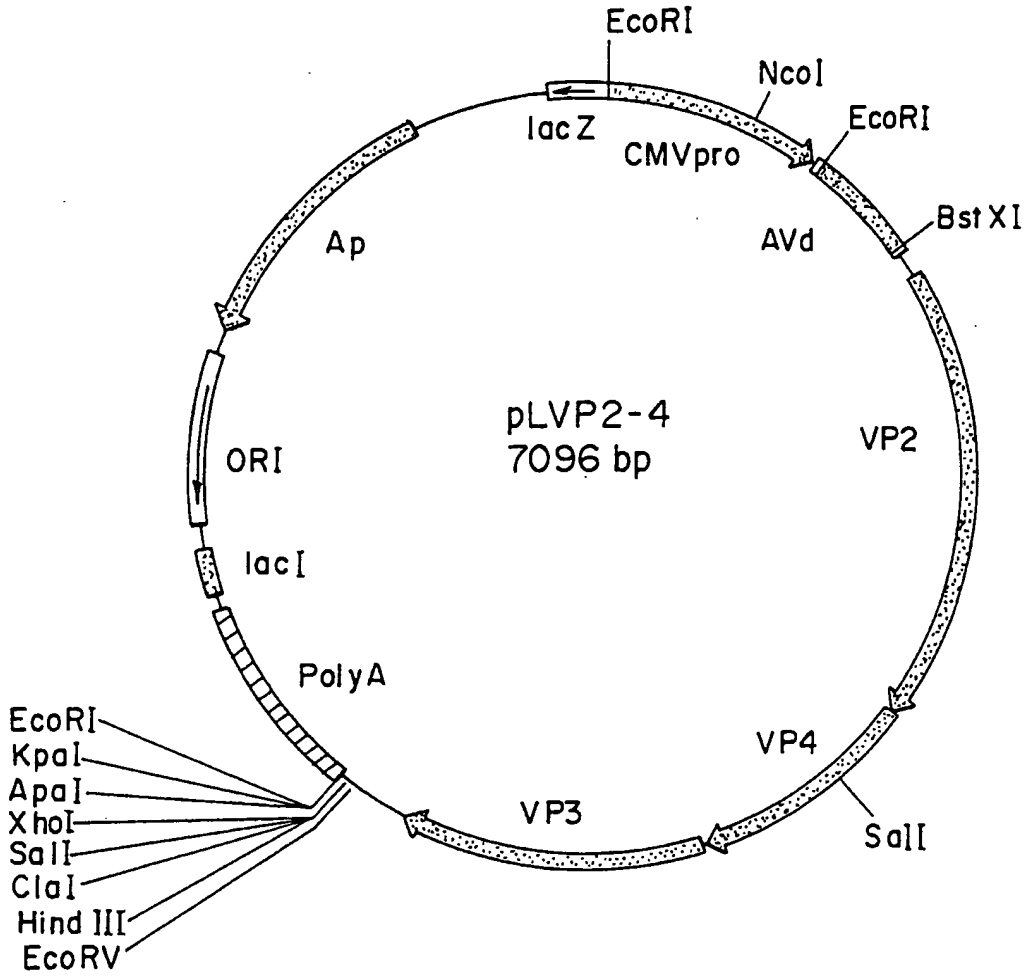
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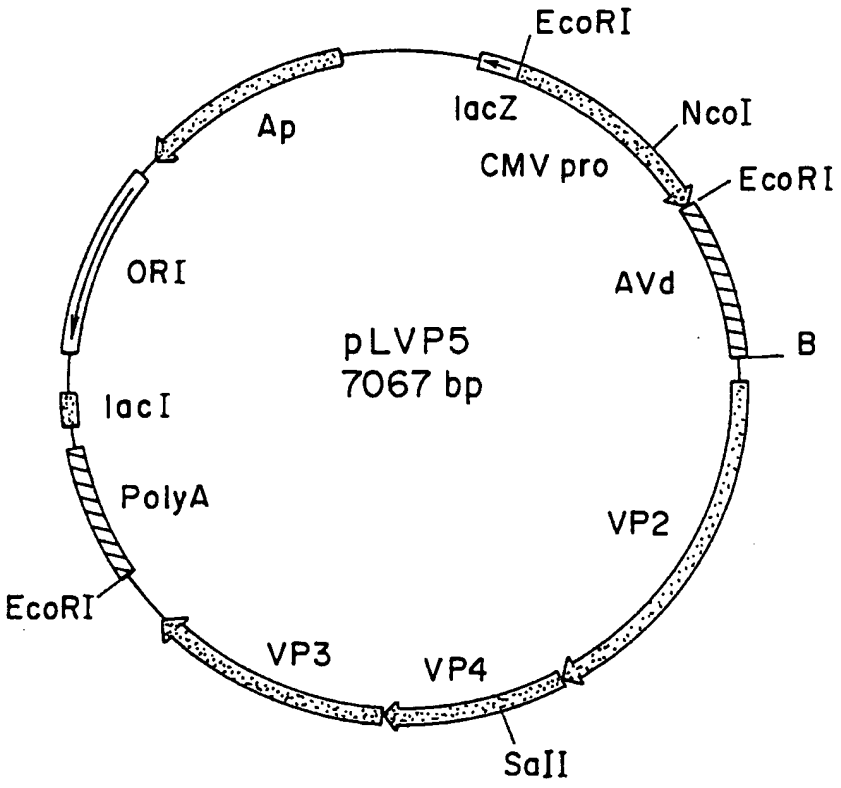
Fig. 5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SEQUENCY LIST

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(i) Characteristics of the sequency:
(A) Length: 1356 bp
(B)Type: nucleic acid

(C) Fibre type:

(D) Topology:

(ii) Molecule type: DNA (genomial)
(vi) Original source:

(A) Organism: IBDV GP40 strain
(ix) Characteristics:
(A) Name/key:
(B)Position: 1....1320

(xi) Description of the sequency No 1:

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(2) DATA OF THE SEQUENCY NO 2:

(i) Characteristics if the sequency:
(A) Length: 452 amino acids
(B) Type: amino acid
(ii) Molecule type: protein

(vi) Original source:

(A) Organism: IBDV GP40 strain

(ix) Characteristics:

(A) Name/key: VP2 antigen-protein
(B) Position: 1....452
(xi) Description of sequency No 2:

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IR